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Running title: Geographic genetic structure of Iberian columbines

ORIGINAL RESEARCH ARTICLE

GEOGRAPHIC GENETIC STRUCTURE OF IBERIAN COLUMBINES (GEN. *AQUILEGIA*)

J. L. Garrido^{1*}, J. M. Alcántara², P. J. Rey², M. Medrano¹, J. Guitián⁴, M. C. Castellanos³, J. M. Bastida², R. Jaime², and C. M. Herrera¹

1 - Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (EBD-CSIC). Avenida Américo Vespucio, s/n, 41092, Sevilla, Spain.

2 - Depto. Biología Animal, Biología Vegetal y Ecología. University of Jaén. 23071, Jaén, Spain.

3 - School of Life Sciences, University of Sussex, Brighton, BN1 9QG, UK.

4 - Depto. Botánica. University of Santiago de Compostela. 15782, Santiago de Compostela, A Coruña, Spain.

*Corresponding author: José L. Garrido, e-mail: jlgarrido@ebd.csic.es, Tel: +34954466700, Fax: + 34954621125.

ABSTRACT

Southern European columbines (genus *Aquilegia*) are involved in active processes of diversification and the Iberian Peninsula offers a privileged observatory to witness the process. Studies on Iberian columbines have provided significant advances on species diversification, but we still lack a complete perspective of the genetic diversification in the Iberian scenario. This work explores how genetic diversity of the genus *Aquilegia* is geographically structured across the Iberian Peninsula. We used bayesian clustering methods, Principal Coordinates Analyses and NJ phenograms to assess the genetic relationships among 285 individuals from 62 locations and detect the main lineages. Genetic diversity of Iberian columbines consists of 5 geographically structured lineages, corresponding to different Iberian taxa. Differentiation among lineages shows particularly complex admixture patterns at Northeast and highly homogeneous towards Northwest and Southeast. This geographic genetic structure suggests the existence of incomplete lineage sorting and interspecific hybridization as could be expected in recent processes of diversification under the influence of Quaternary postglacial migrations. This scenario is consistent with what is proposed by the most recent studies on European and Iberian columbines, which point to geographic isolation and divergent selection by habitat specialization as the main diversification drivers of the Iberian *Aquilegia* complex.

Keywords: *Aquilegia*, AFLP, spatial genetic structure, Iberian Peninsula, diversification, Quaternary postglacial migrations.

INTRODUCTION

Over the last four decades, research on adaptive radiations of Angiosperms favored the view that they were mainly driven by processes of sympatric speciation through pollinator-mediated reproductive isolation, so with phenotypic differentiation mostly involving reproductive traits. The recent diversification of the genus *Aquilegia* in North America strongly supported this view (Hodges and Arnold 1994; Hodges 1997; Whittall and Hodges 2007; Hodges and Derieg 2009). However, recent studies on the Euroasiatic part of the genus have challenged the generality of this perspective, showing that its diversification has been mainly driven by processes of allopatric speciation through geographic isolation and habitat specialization, and mostly involving vegetative traits (Medrano et al. 2006; Alcántara et al. 2010; Bastida et al. 2010; Garrido et al. 2012; Fior et al. 2013; Lega et al. 2014).

These recent studies have also revealed that the endemic taxa from Southern European mountain systems are involved in probably the most active stages of this genus' diversification, and point to the Iberian Peninsula as an optimal natural laboratory for deepening in the study of the European radiation of *Aquilegia*. In fact, studies on Iberian columbines show that some of the differentiation patterns in vegetative traits observed nowadays can be explained by the same divergent selection pressures that currently occur in different habitats (Alcántara et al. 2010). Moreover, vegetative traits of these columbines exhibit higher evolutionary potential than floral traits (Castellanos et al. 2011; Alcántara et al. 2014) and show higher adaptability in defense against herbivores (Jaime et al. 2013), irradiance and water stress (Jaime et al. 2014), and soil properties tolerance (Bastida et al. 2014). In general, these findings agree with the expectations on the divergence drivers of the European columbines (Bastida et al. 2010; Fior et al. 2013).

These studies have provided relevant results working with some Iberian species in particular, and in some specific geographic areas. Nonetheless, it becomes necessary to

provide a complete perspective of the Iberian divergence process by considering all its taxonomic and environmental diversity as a whole, since it may provide interesting keys on *Aquilegia* diversification hardly to detect otherwise. This work explores how genetic diversity of the Iberian *Aquilegia* taxa is geographically structured across the Iberian Peninsula. Given its high habitat heterogeneity (precisely one of the factors shaping the pattern of diversification proposed for Southern European columbines; Bastida et al. 2010; Fior et al. 2013), this geographic context allows us to assess the influence of geographic isolation and habitat specialization on the establishment of the geographic patterns of genetic variation that canalize taxonomic diversification. Besides, a great part of the divergence time of southern European columbines (< 1.7 my; Bastida et al. 2010) overlaps with the period of stronger Quaternary climatic oscillations (0.7 my; Taberlet et al. 1998; Kadereit et al. 2004). Thus, given the critical influence of these events on the settlement of current European flora, this work may provide interesting insights on overall diversification patterns of European plants.

Recent studies on geographic structuring of genetic variation on European columbines distributed across the European Alpine System (Lega et al. 2014) and central Mediterranean islands (Garrido et al. 2012) have revealed strongly structured spatial genetic patterns. These kinds of patterns, when coupled with limited dispersal abilities, like in *Aquilegia* (Hodges and Arnold 1994; Strand et al. 1996), are often associated with geographic isolation scenarios with restricted gene flow between populations and subsequent population differentiation by genetic drift (see Edh et al. 2007).

Spatial patterns of genetic variation in recently radiated groups are often particularly complex mostly due to their high levels of incomplete lineage sorting (Pamilo and Nei 1988; Maddison and Knowles 2006; Joly et al. 2009). This circumstance may become further ‘blurred’ by the high interfertility of these taxa (Prazmo 1965; Taylor 1967). Therefore, these groups usually exhibit uncoupled patterns of genetic and phenotypic differentiation that

generate discrepancies between taxonomy and phylogeny (Wang et al. 2005; Whitfield and Lockhart 2007; Parks et al. 2009; Rymer et al. 2010). Accordingly, the diversification of *Aquilegia* has generated a high number of taxa with low genetic divergence but that exhibit clear phenotypic differences (Hodges and Arnold 1994; Ro and McPheron 1997; Whittall et al. 2006; Whittall and Hodges 2007). Thus, predefined taxonomic entities may not agree with the genetic structure of the group, hindering traditional approaches to the study of spatial genetic structure based on a priori defined populations and species. To avoid this problem we use analytical approaches that do not consider any a priori taxonomic affiliation of the studied populations.

Specifically, the following questions are addressed: (i) How are the Iberian columbines structured genetically across the Iberian Peninsula?; (ii) How does the genetic structure found match with current taxonomy?; (iii) Which may be the main factors shaping this geographic genetic structure?; and, finally, (iv) Are these results compatible with the scenario of divergence by geographic isolation and habitat specialization recently proposed for the European columbines?

METHODS

Study species and sites

Columbines are perennial herbs consisting of an erect rhizomatous stem growing from a basal rosette with bi- or tri-ternate compound leaves. Hermaphrodite, commonly pendant flowers, are radially symmetrical and exhibit five sepals and five petals, each petal consisting of a flat limb and a backwardly directed spur. Basal rosettes grow during April-May and develop one or several inflorescences flowering from May to July, or even August in the case of high altitude species (Cullen and Heywood 1964; Whitemore 1997; Nold 2003). They occur from sea level to 2500 m a.s.l.

Three species inhabit the Iberian Peninsula: *A. vulgaris* (with 4 subspecies), *A. pyrenaica*

(with 4 subspecies), and *A. viscosa* (with 1 subspecies) (Díaz 1986). Out of the 4 *vulgaris* subspecies, *Avv* (see Figure 1 for distribution details and names abbreviations) is widely distributed across the Iberian Peninsula, whereas *Avd*, *Avn*, and *Avp* are, respectively, endemic to the Northwestern mountain systems, Sierra Nevada, and the Ports of Tortosa-Beceite. A fifth *vulgaris* subspecies, *A. v. hispanica* (*Avh*), is questioned as a well differentiated taxon (Díaz 1986). The *pyrenaica* subspecies, *App*, *Apg*, *Apd*, and *Apc* are, respectively, narrow endemics to the Pyrenean Range, Sierra de Guara, Cantabrian Range, and Sierra del Pozo. Finally, the only *viscosa* subspecies, *Avi*, inhabits the eastern Pyrenean Range. *A. vulgaris* occurs in moist developed soils near water streams, damp forest gaps and alpine meadows, while *A. pyrenaica* and *A. viscosa* inhabit rocky limestone outcrops, cliff bases and stony alpine meadows with shallow calcareous soils (Díaz 1986).

Plant material was collected between 2003 and 2009, from 285 wild individuals at 62 Iberian locations. Figure 1 and Table 1 show geographic details of each location and their attributed taxa. Our sampling strategy was designed to cover most distribution areas of the different *Aquilegia* taxa across the Iberian Peninsula, balancing genetic and geographic diversity. To facilitate visualization and analysis, the whole covered area was divided in four geographic areas: Northwest, Northeast, Southeast, and South, as shown in Figs. 1 and 5.

DNA extraction and AFLP protocol

Fresh young leaves were immediately desiccated after collection in the field and stored with silica gel until DNA was processed. Total genomic DNA was extracted from approximately 30 mg of dried leaf material (previously homogenized with a Retsch MM 200 mill) by means of the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and using Qiacube extraction robot (Qiagen, Valencia, CA, USA). Amplified fragments were obtained following the protocol by Vos et al. (1995) with some modifications related to the use of fluorescent dye-labeled selective primers. *EcoRI* and *MseI* were used as rare and frequent cutter enzymes,

respectively. Digested DNA was ligated to the *Eco*RI and *Mse*I adapters. Fragments were selectively amplified with *Eco*RI and *Mse*I primers, bearing one selective nucleotide. Final selective amplifications were carried out using *Eco*RI and *Mse*I primers with three selective nucleotides (see table in Online Resource 1).

Twenty-four primer combinations were preliminarily assayed for selective amplification on a random subsample of 16 individuals. Eight primer combinations were finally selected based on fragment abundance and polymorphism. Fragment detection was performed by means of an ABI PRISM 3100 DNA automatic sequencer (Life Technologies, Carlsbad, CA, USA). Fragments presence on each individual was manually scored with GeneMapper v.4 (Life Technologies, Carlsbad, CA, USA).

Reproducibility and reliability of AFLP fragments were assessed by replicating 16 individuals. The replication procedure was carried out including DNA isolations, since repeating just the process of AFLP production does not generate true replicates (Bonin et al. 2007; Holland et al. 2008). The scoring error rate was very low and ranged, among primer combinations, between 0.54 and 1.90 % (mean 1.34 %, Online Resource 1) indicating the suitability and repeatability of our dataset.

AFLP data analyses

To avoid possible fragment size homoplasy, only 150-500 bp size fragments were finally considered since preliminary assays indicated that smaller fragments were prone to it (Vekemans et al. 2002; Herrera and Bazaga 2009; Garrido et al. 2012).

Genetic diversity was assessed by the percentage of polymorphic loci (PPL), the expected heterozygosity (H_e ; Nei 1987), and the unbiased H_e for small sample sizes (Nei 1978). Since in dominant markers band presence may reflect either homozygosity or heterozygosity, the null allele frequency has always to be estimated. Thus, genetic parameters based on allelic frequencies, like H_e , are difficult to assess, existing different methods to estimate them (Bonin

et al. 2007; Meudt and Clarke 2007). Here, we have used a Bayesian method with non-uniform prior distribution of allelic frequencies, assuming Hardy-Weinberg Equilibrium, as implemented in AFLPSurv v.1.0 (Vekemans et al. 2002). Calculations were performed on 4-5 individuals with complete genotypic profiles for each location.

Long-term isolated populations accumulate rare markers by mutations, thus, in order to provide indirect estimates of location divergence and isolation, we further calculate three parameters of markers rarity: the number private markers (markers present in only one population, NPM), the number of diagnostic markers (private markers present in every individual in a population, D), and the frequency-down-weighted marker values (DW), which may be used as a standardized measure of divergence and long-term isolation (Schönswetter and Tribsch 2005). At each location, the average number of markers per individual (NMI) and the number of fixed markers (NFM) were also calculated. Excepting DW, obtained with AFLPdat (Ehrich 2006), these parameters were calculated with FAMD v.1.31 (Schlüter and Harris 2006).

The underlying genetic structure among individuals and locations was analyzed under three different approaches. We firstly use a Bayesian method to group individuals into natural genetic groups (K) based on their individual multilocus profiles, as implemented in the software STRUCTURE v.2.3.3 (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009). By means of Markov chain Monte Carlo algorithms, it calculates the posterior probability of membership of an individual to a number of K groups without prior information on their taxonomic identity and population origin. Analyses were performed using an admixture ancestry model with recessive alleles and correlated allele frequencies among populations. Thirty independent runs were performed for each K value (from 1 to 21), each with a 10^5 MCMC iterations length after a burn-in period of $5 \cdot 10^4$ steps. To evaluate the similarity among runs and whether clustering solutions for each K value were consistent, we

calculated their similarity coefficients using the R-script STRUCTURE-SUM-2009 (Ehrich et al. 2007). To detect the true number of genetic groups we used the modal ΔK parameter, proposed by Evanno et al. (2005). Extraction of key results, integration of the outcomes from different runs and representation of STRUCTURE outputs were performed by using, respectively, STRUCTURE HARVESTER (Earl and vonHoldt 2012), CLUMPP v.1.1.2 with Greedy algorithm (Jakobsson and Rosenberg 2007), and DISTRUCT v.1.1 (Rosenberg 2004).

To confirm the distribution of individuals among genetic groups and verify the adscription of individuals to the clusters revealed by STRUCTURE, we further performed another different Bayesian clustering analysis by means of BAPS v.6.0 (Corander et al. 2003; Corander and Marttinen 2006). It consisted of an initial individual mixture analysis with fixed $K = 5$, iterated 20 times to assess the consistency among simulations. And then, an admixture analysis with 100 iterations to estimate the overall admixture coefficients, estimating with 100 replicates the admixture of 200 reference individuals.

We also explored the genetic structure without the population genetics assumptions underlying the former Bayesian approach by means of a principal coordinates analysis (PCoA; Krzanowski 1990) using FAMD v.1.31 (Schlüter and Harris 2006). This analysis was based on Jaccard distances among individuals, since in AFLP markers, shared absences (null alleles) are particularly susceptible to homoplasy, being thus dissimilarity measures that do not take into account them (like Jaccard and Nei-Li distances) the most appropriate (Meudt and Clarke 2007).

A third approach was to depict genetic relationships among locations and individuals by constructing an unrooted Neighbor-Joining (NJ) phenogram based on the Nei-Li distances among individuals (Nei and Li 1979). 2000 bootstrap replicates, and their corresponding Nei-Li distance matrices and NJ trees, were obtained by using respectively the SEQBOOT, RESTDIST, and NEIGHBOR modules of PHYLIP v.3.6. software (Felsenstein 2005).

Finally, a 50% majority rule consensus tree was constructed with PAUP* v.4.0a152 (Swofford 2002).

RESULTS

We consistently and unambiguously scored 474 loci (453 polymorphic) for 285 individuals from 62 *Aquilegia* locations sampled across the Iberian Peninsula. Each primer combination resolved 59.37 loci on average (see table in Online Resource 1).

Genetic diversity and markers rarity across locations

Estimates of genetic diversity across locations, even the unbiased estimate for low sample sizes, revealed overall low values, with PPL, H_e , and uH_e averaging 26.09 (± 2.54), 0.0705 (± 0.0062), and 0.0787 (± 0.0069), respectively (Table 2). Genetic diversity ranged from lowest values at PD2 (PPL: 21.3), AP3 (H_e : 0.0500) and AP4 (uH_e : 0.0562), to highest values at AVA (PPL: 32.7, H_e : 0.1115, uH_e : 0.1274). Parameters of markers rarity revealed that APG, specially, but also PAU, AC4, and PD2 showed highest values of divergence and long-term isolation (Table 2). They exhibited highest numbers of private and diagnostic markers (NPM (D): 4 (4), 3 (1), 6 (2), and 2 (1), respectively) and the highest DW estimates (35.99, 25.75, 25.41, and 24.61, respectively).

Geographic structure of genetic variation

The most likely STRUCTURE partition involved the existence of 5 genetic lineages ($\Delta K = 12.96$, $Pr|ln L = -28366.59$; Online Resource 2) in the Iberian Peninsula (Figure 2), which will be hereafter referred to as colors red, green, purple, blue, and orange. All 30 runs gave consistent similarity results and individuals were clearly assigned to nonempty groups.

Lineages hosted nearly homogeneous genetic compositions for all locations, although some of them showed varying admixture levels. BAPS showed an virtually identical distribution of individuals among genetic groups (Online Resource 3) than that of STRUCTURE. The only incongruence was that, instead of assigning the majority ancestry contribution of APG to the

blue lineage like STRUCTURE does, it assigned it to the purple one.

PCoA analyses also identified these same five genetic lineages, as well as individuals in displaced positions from the cluster pure core reflecting different admixture levels (Figure 3). The first axis explained the 18.63 % of total genetic variation and segregated blue individuals from the rest, which were in turn further structured by the secondary axis (12.91 %) in three clouds, mostly corresponding to the green, orange, and red lineages. The third axis (8.46 %) revealed a clear segregation of purple individuals. Three small sub-clouds, coinciding with APG, PAU, and PD2 locations, may also be identified. The unrooted NJ phenogram was also in agreement with the results from the Bayesian and PCoA approaches (Figure 4). It resolved with a high bootstrap support (95.35%) two major clades, one with the locations from the blue and purple lineages (BP clade) and other with the locations from the green, red, and orange lineages (GRO clade). Further, the clades corresponding to the five lineages were also well supported (blue: 100 %, purple: 84.25 %, green: 76.20 %, and orange: 75.25 %), although the clade matching to the red cluster received a lower support (66.90 %).

Complex admixture patterns could also be detected here with PXV, AVA, VLM, VRG, and VLB presenting undefined positions within the GRO clade. Additionally, PAU and APG represented independent subclades within the BP clade. Interestingly, both locations, together with PD2 which represents an independent blue subclade, also represent separated sub-clouds in the PCoA and are highlighted as highly diverged and long-term isolated localities.

Geographically, the green, red, and orange lineages occur in all explored areas, while purple occurs only in the East (Figures 5b, c) and blue only in the North (Figures 5a, b). Pure red locations dominate in the Northwest (Cantabrian Range, Galaico-Leonés Massif and pre-coastal mountains; Figure 5a). The red lineage also occurs in some northeastern locations, although exhibiting varying admixture levels (Figure 5b). Purely or predominantly green locations occur in the Southeast (Sierras de Cazorla, Segura y Las Villas, Figure 5c), while

the orange lineage predominates in the South (Sierra Nevada and Sierra de Tejada, Figure 5d). Purely or predominantly blue locations mainly occur in the Northeast (Pyrenean Range, Figure 5b), but this lineage also appears in the Northwest (central Cantabrian Range, Figure 5a). Finally, purely purple locations occur in a small southeastern area (Figure 5c), and beyond this, purple lineage abounds in two locations far separated to the north (Figure 5b): PAU in the eastern Iberian System and APG in the southern Pyrenean Range, both also segregated in the PCoA and NJ ordinations and highlighted as long-term isolated locations. Interestingly, BAPS highlighted the high purple contributions of both locations (Online resource 3).

Although different levels of lineages admixture occur throughout, all analytical approaches reflected that locations at the Iberian Northeast host the most remarkable levels. PXV, VLM, APG, AVA, and VRG presented admixtures of up to 5 lineages (Figure 2).

Genetic diversity, markers rarity and differentiation among lineages

Estimates of genetic diversity and markers rarity supported and complemented the genetic structure here defined. Lineages differed significantly in genetic diversity (PPL: $F_{4,55} = 0.03$, $p = 0.0061$; H_e : $F_{4,55} = 2.91$, $p = 0.0294$) and markers rarity (NFM: $F_{4,55} = 2.68$, $p = 0.0404$; DW: $F_{4,55} = 3.66$, $p = 0.0102$), with blue lineage hosting the less genetically diverse and most long-term isolated locations. PPL and H_e in the blue lineage (22.8 and 0.0557, respectively) were lower than those in the red (26.3 and 0.0714; Tukey's Test: $p = 0.0056$ and $p = 0.0070$) and green ones (26.9 and 0.0749; $P < 0.0001$ and $p = 0.0517$). PPL in the blue lineage was also lower than in the purple one (26.4, $p = 0.0202$). Similarly, DW of the blue lineage (17.96) was significantly lower than that of green (9.97; $p = 0.01924$) and red (8.17; $p = 0.0014$). In addition, the genetic structure model defined by STRUCTURE revealed overall high F_{ST} values for all lineages (Purple: 0.49, Red: 0.50, Green: 0.53, Orange 0.52, Blue: 0.49).

DISCUSSION

The overall genetic diversity of *Aquilegia* across the Iberian Peninsula is divided into 5 geographically structured lineages that, as will be later detailed, largely agree with the main Iberian taxonomic entities (Díaz 1986). This geographic structuring becomes reinforced by the differences among lineages in genetic diversity and isolation, their high F_{ST} values, and the low overall levels of genetic diversity, which further suggest low rates of gene flow for the whole system.

Strongly spatially structured genetic patterns have been observed in other Mediterranean species complexes, like *Nigella arvensis* (Bittkau and Comes 2005), *Viola suavis* (Mereda 2011), and *Anthemis secundiramea* (Lo Presti et al. 2011). Interestingly, strong geographic isolation and low genetic diversity within populations seem to be the rule in columbines, as also detected in North American (Strand et al. 1996) and in the Sardinian species (Garrido et al. 2012). In the northern Mediterranean Basin, these strong geographic structurings have been frequently attributed to its complex quaternary paleographic and paleoclimatic history (Bennett et al. 1991; Medrano and Herrera 2008). Accordingly, the heterogeneous Iberian topography and climate, together with the limited dispersal ability of *Aquilegia* (Hodges and Arnold 1994; Strand et al. 1996), seem to have promoted a fragmented distribution, with a concomitant genetic isolation, which have favored eventual processes of species diversification through allopatric speciations (Thompson 2005). Furthermore, and given that the Iberian *Aquilegia* radiation started 1.77 my ago (Fior et al. 2013), thus excluding major paleogeographic events as possible drivers, quaternary climate oscillations most probably played the main role by generating secondary contacts through range expansions and/or retractions of previously isolated, related taxa (Comes and Kadereit 2003; Kadereit et al. 2004), something, as will be later discussed, also suggested by our data.

Before discussing how this pattern is geographically structured, and far from pretending any kind of taxonomic assessment (something this work is not specifically designed to), we describe below how the geographic genetic structure here found matches current Iberian *Aquilegia* taxonomy, since this can support later discussion.

Correspondence with current Iberian *Aquilegia* taxonomy

Each of the 5 lineages detected corresponded to a single taxonomic identity. The vast majority of locations grouped in the green, red, orange, blue, and purple lineages exhibited pure genotypes and are attributed to *Avv*, *Avd*, *Avn*, *App*, and *Apv*, respectively (see Table 1 and Figure 2). In addition, the NJ phenogram revealed two major, well supported clades that split the localities attributed to *A. pyrenaica* and those attributed to *A. vulgaris*. These major clades were later divided according to the 5 lineages detected (Figure 4).

Apart from these pure locations attributed to the main Iberian *Aquilegia* taxa, locations with undifferentiated (PD2, AV2, and AV3) or complex (VLM, VLB, VRG, and APG) genotypic profiles are ascribed to the less frequent taxa (*Apd*, *Apg*, *Avp*, and *Avi*). But, there still remains a number of locations (namely, PAU, A00, AV9, CSC, ACN, ACB, ACS, AMA, SIL, PD1, AVA, and PXV) attributed to taxa whose associated lineages do not correspond with those suggested here by their genotypic profiles. This generates an uncoupling between genetic and taxonomic diversity that complicates traditional intra and inter-specific analyses of genetic variation based on a priori well-defined populations and species.

PD2 is an almost pure blue (*App*) location represented by a blue subgroup in the PCoA and NJ ordinations, and it is described as *Apd*, a narrow endemic to the Cantabrian Range. AV2 and AV3 do not differ genetically from the rest of green locations attributed to *Avv* and are attributed to *Avh*, a currently questioned *vulgaris* subtaxa (Díaz 1986). VLM, VLB, and VRG are characterized by complex admixtures and undefined positions among the *vulgaris* groups in the PCoA and NJ ordinations. They are attributed to *Avi*, the third, less frequent Iberian

species, endemic to the Iberian Northeast.

APG and PAU, although also host *vulgaris* genotypes, are characterized by the admixture of blue (*App*) and, particularly of purple (*Apc*) lineages. PCoA and NJ phenogram place them as well-differentiated *pyrenaica* locations, next to *Apc*. APG is described as *Apg*, a narrow endemic to the southern Pyrenean Range, and PAU is described as *Avp*, an extremely narrow endemic to the eastern Iberian System. Its ascription as a *vulgaris* taxa, given also its marked morphological differences with *vulgaris* (Díaz 1986, Martinell et al. 2011), does not agree with our data, in the light of which, it could represent an independent *pyrenaica* taxa, like APG. Its *vulgaris* ascription has been attributed to a possible herbarium confusion when firstly described by Font Quer in 1920 (Martinell et al. 2011).

A00, AV9, CSC, ACN, ACB, and ACS do not differ genetically from the green locations attributed to *Avv*, and are ascribed to *Apc*. Their frequent intermediate morphological traits (M. Medrano and C. Herrera, personal observation), something coinciding with a minor but consistent purple contribution (Online Resource 4), are probably beneath this apparent misleading affiliation. AMA, SIL, and PD1 do not differ genetically from pure red locations attributed to *Avd*, which seems to mismatch with their ascriptions as *App*, *Avv*, and *Apd*, respectively. At PD1, the small individual size may have mislead its classification as *Apd*, the smallest Iberian *Aquilegia* taxa together with *Avp*. As earlier commented for other northeastern locations, AVA and PXV are characterized by complex admixtures and undefined positions among the *vulgaris* groups in the PCoA and NJ ordinations. AVA is described as *Avv*, although genetically different from locations attributed to *Avv*; and PXV is an undetermined location very similar genotypically to VLM, described as *Avi*.

This uncoupling between genetic and taxonomic structure, eventually leading to numerous gene trees/species trees incongruences, is common in rapidly radiating plants (e.g. Wang et al. 2005, Whitfield and Lockhart 2007, Parks et al. 2009, Rymer et al. 2010) and reflects the

recent and rapid nature of the *Aquilegia* radiation, particularly in Southern Europe (Bastida et al. 2010, Fior et al. 2013). Both in North America (Hodges and Arnold 1994, Ro and McPherson 1997, Whittall et al. 2006, Whittall and Hodges 2007, Fior et al. 2013) and Eurasia (Bastida et al. 2010, Fior et al. 2013), *Aquilegia* diversification has led to a high number of low genetically divergent or hybrid taxa that exhibit marked morphological differences.

Differentiation among lineages

Regarding how this pattern is geographically structured, the mosaic of complex genetic profiles (i.e. complex admixture patterns and undefined positions in PCoA and NJ ordinations) represented by the northeastern locations contrasts with the numerous genetically defined southern/southeastern and northwestern locations. This depicts a whole Iberian diversification scenario where lineages remain highly undefined at the Northeast (a hotchpotch of all Iberian lineages) and seem more differentiated both to the South/Southeast and, particularly, to the Northwest. Hence, our data point to the Iberian Northeast as a particularly active area in the Iberian *Aquilegia* diversification, while towards southern and northwestern mountain systems the process turns less active. Not in vain, some genetically complex northeastern locations define different narrow endemic taxa (*Avi* at VLM, *Apq* at APG, and *Avp* at PAU), which in turn, further coexist with some genetically defined taxa like *App*. Differently, the other areas are dominated by fewer and better defined taxa (*Avd*, at Northwest; *Avv* and *Apq*, at Southeast; and *Avn*, at South). The Iberian Northeast has also been highlighted as a particularly diverse area for other plants which has been attributed to its role as a contact zone with taxa from the other side of the Pyrenean range due to postglacial migrations and to its role as quaternary glacial refugium (see e.g.: Olalde et al. 2002, on white oaks; Magri et al. 2006, on European beeches; and Cottrell et al. 2005, on black poplars).

Out of all different links among Iberian lineages, those among green, red, and orange ones (*Avv*, *Avd*, and *Avn*, respectively) are particularly frequent and can be detected throughout the

Iberian Peninsula, especially in the Northeast. These lineages (regarded as subspecies of *A. vulgaris*) are older than blue and purple (regarded as subspecies of *A. pyrenaica*), since *A. pyrenaica* is a more recently derived taxon (Bastida et al. 2010). This, together with their ubiquity, may reflect a certain degree of incomplete lineage sorting (ILS, hereafter) among them. Which may be also supported by the southern orange/red and southeastern green/orange links, where distances and habitats separating both lineages make hybridization highly difficult. Given the recent and rapid nature of *Aquilegia* diversification (particularly of its southern European clade; Bastida et al. 2010, Fior et al. 2013), high ILS levels would imply that genetic drift is unlikely to have had time, even dealing with these oldest lineages, to bring loci to fixation before subsequent divergence episodes (Pamilo and Nei 1988; Maddison and Knowles 2006). Interestingly, localities with admixtures exclusively involving these lineages hosted less fixed loci than the rest (82.43 ± 11.34 vs. 88.59 ± 9.13 ; $F_{1,58} = 4.68$, $p = 0.0345$). Despite this, since ILS effects are difficult to distinguish from those of hybridization events, these admixtures may also reflect hybridization, as will be discussed later.

Links involving blue and purple lineages, although less frequent, also reveal interesting cues on the Iberian diversification of *Aquilegia*, probably related to Quaternary range shifts. The blue lineage (*App*) is limited to some areas across northern ranges, while the purple one (*Apc*) is restricted to a small southeastern area. Thus, their co-occurrence at some northeastern locations, particularly relevant at PAU and APG, makes them stand as possible footprints of a past coexistence of blue and purple lineages at northeastern mountain systems. Interestingly both are highly isolated and genetically differentiated locations defining the potentially hybrid taxa *Avp* and *Apg*, respectively. Purple lineage would thus have been distributed northwards, across eastern mountain systems, and later retracted to its current restricted southeastern Prebetic distribution (not in vain, a putative quaternary refugium for many plants; Médail and Diadema 2009). And, on the other hand, blue lineage would have reached eastern mountains

systems, facing southwards the strong barrier represented by the Ebro river depression. These kinds of range shifts are well known to influence current geographical genetic patterns of many western European floras (Martín-Bravo et al. 2010; Nieto 2014), particularly of endemic mountain species (Kropf et al. 2003). They were generated by climate oscillations that became particularly pronounced in the last 0.7 my (Taberlet et al. 1998; Kadereit et al. 2004). Therefore, given that European *Aquilegia* have diversified in the last 1.7 my (Bastida et al. 2010), they have surely exerted an exceeding influence due to their coincidence along a large part of the Iberian *Aquilegia* divergence time.

Apart from these links among *vulgaris* and among *pyrenaica* lineages, admixtures of both were also detected at the northeast and southeast, probably indicating interspecific processes of hybridization. Northeastern APG, VLM, PAU, and PXV locations consist of varying genotypic contributions of different *pyrenaica* and *vulgaris* lineages and define the potentially hybrid taxa *Ap_g*, *Av_i*, and *Av_p*, respectively (PXV was undetermined). Given that gene flow increases the sharing of ancestral polymorphisms (Wang et al. 1997; Machado et al. 2002, Maddison and Knowles 2006), like those retained by ILS, these northeastern hybridizations could explain why in this area the process of lineage sorting seems in more incomplete stages and is more persistent than at other Iberian areas. In the southeast, green (*Av_v*) and purple (*Ap_c*) groups are parapatrically distributed with a clear contact zone where potentially introgressive hybridization may be taking place. Not in vain, when this area was analytically focused, green localities closer to purple group revealed minor but consistent purple contributions (Online Resource 4). Interestingly, these locations exhibited intermediate morphological traits between both species (see discussion on correspondence with taxonomy).

Conclusions

This highly geographically structured pattern is consistent with the current knowledge on European and Iberian *Aquilegia* taxa. Recent studies on the whole genus (Fior et al. 2013)

and, specifically, on Euroasiatic taxa (Bastida et al. 2010) suggest that divergence of southern European lineages is initially shaped by geographic isolation and habitat partitioning. Further, given that the limited dispersal ability of the genus (Hodges and Arnold 1994; Strand et al. 1996) makes long distance dispersal events unlikely (but see Garrido et al. 2012; Lega et al. 2014), allopatric speciation is proposed to have played a key role in the European *Aquilegia* divergence, particularly, in geographically restricted contexts (Fior et al. 2013) like those corresponding here to the numerous endemic (*Avd*, *Avi*, *Avn*) and narrow endemic (*App*, *Apc*, *Apd*, *Avp*, and *Apg*) taxa inhabiting the Iberian Peninsula.

Regarding the underlying evolutionary drivers of the Iberian *Aquilegia* diversification, several studies have found evidence of how different environmental habitat parameters impose divergent selection patterns on phenotypic divergence and ecotypic differentiation (Alcántara et al. 2010; Jaime et al. 2013; Bastida et al. 2014). Green (*Avv*), purple (*App*), and orange (*Avn*) lineages coexist in the same area, thus surely having experienced the same climate changes over the last millennia. However their different environmental requirements may have determined different effects of climate oscillations on each one, eventually entailing different evolutionary consequences. The widely distributed *Avv* inhabits permanent water courses at lower altitudes, while *Apc* and *Avn* mainly occupy springs in the higher parts of the mountains (Jaime et al. 2013; Bastida et al. 2014). Thus, *Apc* and *Avn* populations may be intrinsically small and isolated regardless of the climatic scenario, while *Avv* can expand and/or retract along the river network according to climatic oscillations.

The pattern here proposed has revealed the effects of Quaternary range shifts, strongly influenced by the high interfertility of these taxa (Prazmo 1965; Taylor 1967). Postglacial migrations and geographic isolations would have occurred over very short time periods thus not giving enough time for the genetic drift to act, which further, may be beneath the ILS detected. These circumstances are responsible of the morphological /genetic uncoupling

found and entangle the Iberian *Aquilegia* diversification, particularly in the Northeast, where many cases of hybridized identities, likely incipient taxa, appear defined as separate species (*Avi*) or subspecies (*Avp*, *Apg*).

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that the research included in this article accomplishes with the ethical standards of the journal and with all legal requirements regarding samples collection. All authors have been informed and consent with this submission. Funding sources have been declared both in Acknowledgements section as well as in the online submission form. The research does not involve human participants and/or animals.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ELECTRONIC SUPPLEMENTARY MATERIAL

Online Resource 1. Details on primers combinations used.

Online Resource 2. Variation in ΔK parameter for the STRUCTURE analysis.

Online Resource 3. Admixture analysis performed with BAPS.

Online Resource 4. STRUCTURE analysis specifically performed on Prebetic locations.

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FIGURE CAPTIONS

Figure 1. Geographic distribution of Iberian columbines (differently delineated areas, as shown in legend) and sampled locations (black dots). Abbreviation of taxa names: *Avv*: *Aquilegia vulgaris* subsp. *vulgaris*; *Avd*: *A. v.* subsp. *dichroa*; *Avn*: *A. v.* subsp. *nevadensis*; *Avp*: *A. v.* subsp. *pau*; *Avh*: *A. v.* subsp. *hispanica*; *App*: *A. pyrenaica* subsp. *pyrenaica*; *Apc*: *A. p.* subsp. *cazorlensis*; *Apg*: *A. p.* subsp. *guarensis*; *Apd*: *A. p.* subsp. *discolor*; and *Avi*: *A. viscosa* subsp. *hirsutissima*. To facilitate visualization, the sampled Iberian Peninsula is divided in four geographic areas (delimited by white lines and shown in detail in Figure 5): Northwest (a), Northeast (b), Southeast (c), and South (d). Taxonomic source: *Flora Ibérica* (Díaz 1986), and www.anthos.es. Base map source: ESRI.

Figure 2. PCoA ordination based on the Jaccard genetic distances among the 276 *Aquilegia* individuals from 62 locations sampled across the Iberian Peninsula. The upper plane is defined by PC1 and PC2 factors and segregated individuals mostly corresponding to the five genetic clusters defined by STRUCTURE (blue, purple, green, orange, and red). The lower plane, defined by PC1 and PC3, segregated blue and purple individuals. Symbols colour refers to the lineages detected by STRUCTURE analyses. Symbols shape indicates different groups of locations as indicated in the legend.

Figure 3. Unrooted neighbour-joining, majority rule 50% phenogram based on the Nei-Li (1979) genetic distances among the 276 *Aquilegia* individuals sampled across the Iberian Peninsula, calculated from 476 AFLP loci. Numbers on basal branches indicate > 60% of node bootstrap support, based on 2000 bootstrap replicates. Values > 70% are shown in bold. Clades are coloured according to the clusters defined by STRUCTURE. Locations acronyms follow Table 1.

Figure 4. STRUCTURE analysis results on the genetic structure of the 62 Iberian *Aquilegia* locations based on 474 AFLP individual multilocus profiles. Results show the individual memberships to each of the 5 genetic clusters detected, with each vertical bar representing an individual multilocus genotype. Colours indicate the most likely ancestry of the cluster from which the genotype or partial genotype was derived. Bars with multiple colours indicate admixtures of genotypes from different clusters. Locations are separated by black lines and their acronyms follow Table 1.

Figure 5. Geographic genetic structure of the Iberian *Aquilegia* locations studied. Pie charts indicate the mean proportion of membership of each local population to the 5 genetic groups detected by STRUCTURE. It is represented separately in the four areas with more coexisting taxa, as indicated in Figure 1, namely, Northwest (a), hosting mostly pure red locations (red locations, hereafter); Northeast (b), with pure blue locations and highly admixed, mainly

red, locations; Southeast (c), mainly green and purple locations; and South (d), mainly orange locations. Note the different scales in panels a-b and c-d.

TABLES

Table 1. Localities details including names, specific locations, mountain systems, and geographic coordinates of the 62 Iberian *Aquilegia* sampling points studied. For each one, an acronym (ID), the number of individuals collected (N), and the currently attributed taxa are also provided. Iberian geographic areas, where we have focused our study (according to Figure 1 and 5), are also indicated. ?: undefined determination. ND: not previously cited. *: collected by MCM (see Acknowledgements) as a *Avv x Avp* hybrid. See Figure 1 for taxa abbreviations.

ID	Locality name	Location (Province)	Mountain System (geographic area)	Lat/Long	Taxa	N
A00	Barranco de La Charca	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9419/-2.8633	<i>Apc?</i>	5
AC1	Barranco de La Canal	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.7855/-2.9557	<i>Apc</i>	5
AC2	Cerrada del Pintor	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8633/-2.9308	<i>Apc</i>	5
AC3	Cerrada del Pintor (Dique)	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8716/-2.9115	<i>Apc</i>	5
AC4	Cerro Cabañas	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8076/-2.9557	<i>Apc</i>	5
AC5	Cerro Cabañas (Norte)	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8129/-2.9554	<i>Apc</i>	5
AC6	La Canaleja	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8718/-2.9103	<i>Apc</i>	5
AC7	Arroyo de La Mesa	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8992/-2.913	<i>Apc</i>	3
ACB	Cuevas Bermejas (Pared)	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9632/-2.8489	<i>Apc?</i>	5
ACN	Charca Norte	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9482/-2.8616	<i>Apc?</i>	1
ACS	Charca Sur	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9431/-2.8627	<i>Apc?</i>	5
AMA	Circo del Mampodre	Macizo del Mampodre (León)	Cantabrian Range (Northwest)	43.0285/-5.1883	<i>App</i>	4
AN1	La Cortijuela I	Sierra Nevada (Granada)	Penibetic Range (South)	37.0744/-3.4736	<i>Avn</i>	5
AN2	La Cortijuela II	Sierra Nevada (Granada)	Penibetic Range (South)	37.0816/-3.4684	<i>Avn</i>	5
AN3	Pradollano	Sierra Nevada (Granada)	Penibetic Range (South)	37.0978/-3.4012	<i>Avn</i>	5
AN4	Fuente Fría	Sierra Nevada (Granada)	Penibetic Range (South)	37.0184/-3.4901	<i>Avn</i>	5

AN5	Aguas Blanquillas	Sierra Nevada (Granada)	Penibetic Range (South)	37.07/-3.4824	<i>Avn</i>	5
AN6	La Maroma	Sierra de Tejeda (Málaga)	Penibetic Range (South)	36.8977/-4.047	<i>Avn</i>	5
AP1	Collado Tortiellas	Central Pyrenean Range (Huesca)	Pyrenean Range (Northeast)	42.7784/-0.5407	<i>App</i>	4
AP2	Candanchú	Central Pyrenean Range (Huesca)	Pyrenean Range (Northeast)	42.7737/-0.5304	<i>App</i>	5
AP3	El Tobazo	Central Pyrenean Range (Huesca)	Pyrenean Range (Northeast)	42.7798/-0.5232	<i>App</i>	5
AP4	Larra	Macizo de Larra-Belagua (Navarra)	Pyrenean Range (Northeast)	42.9681/-0.7677	<i>App</i>	5
AP5	Las Blancas	Central Pyrenean Range (Huesca)	Pyrenean Range (Northeast)	42.7157/-0.5619	<i>App</i>	4
APG	Guara I	Sierra de Guara (Huesca)	Pyrenean Range (Northeast)	42.2993/-0.2223	<i>Apq</i>	5
AV1	Barranco de los Jabalises	Sierra de Segura (Jaén)	Prebetic Range (Southeast)	38.2071/-2.5849	<i>Avv</i>	5
AV2	Cascada de 'El Saltador'	Sierra de Segura (Jaén)	Prebetic Range (Southeast)	38.3234/-2.5418	<i>Avh?</i>	5
AV3	Rio Tus	Sierra de Segura (Jaén)	Prebetic Range (Southeast)	38.3551/-2.5031	<i>Avh?</i>	5
AV4	Cueva del Peinero	Sierra de Las Villas (Jaén)	Prebetic Range (Southeast)	38.1026/-2.8684	<i>Avv</i>	5
AV5	Barranco del Guadalentín	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.898/-2.8378	<i>Avv</i>	5
AV6	Embalse del Aguascebas	Sierra de Las Villas (Jaén)	Prebetic Range (Southeast)	38.0763/-2.8953	<i>Avv</i>	5
AV7	La Cabrilla	Sierra de Segura (Jaén)	Prebetic Range (Southeast)	37.9261/-2.7867	<i>Avv</i>	5
AV8	Fuente de la Reina	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9436/-2.8327	<i>Avv</i>	5
AV9	Covacho del Aire	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9514/-2.8602	<i>Apc</i>	5
AVA	Valle Aisa	Central Pyrenean Range (Huesca)	Pyrenean Range (Northeast)	42.7375/-0.5885	<i>Avv</i>	4
AZ1	Cuevas de La Mesa	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8968/-2.9185	<i>Apc</i>	3
CCB	Cuevas Bermejas (Cer.)	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9631/-2.8534	<i>Avv</i>	5
CSC	Charca Sur (Cercado)	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.9453/-2.8619	<i>Apc</i>	5
D01	Puerto de Vegarada	Macizo Asturleonés (León)	Cantabrian Range (Northwest)	43.039/-5.4694	<i>Avd</i>	5
D02	Aula de la Natulaleza	Sierra de O Courel (Lugo)	Macizo Galaico-Leonés (Northwest)	42.558/-7.1125	<i>Avd</i>	5
D03	La Uña	Macizo Asturleonés (León)	Cantabrian Range (Northwest)	43.055/-5.1319	<i>Avd</i>	5
D04	Puerto de Ventana	Macizo Asturleonés (León)	Cantabrian Range (Northwest)	43.0641/-6.0058	<i>Avd</i>	5
D05	Cabaña Arce (Moreda)	Sierra de O Courel (Lugo)	Macizo Galaico-Leonés (Northwest)	42.6254/-7.1102	<i>Avd</i>	5
D06	Isoba	San Isidro (León)	Cantabrian Range (Northwest)	43.049/-5.319	<i>Avd</i>	5
D07	Peñalba de Santiago	Montes de León (León)	Macizo Galaico-Leonés (Northwest)	42.4387/-6.5516	<i>Avd</i>	5

D08	La Aquiana	Montes de León (León)	Macizo Galaico-Leonés (Northwest)	42.4479/-6.5991	<i>Avd</i>	5
D09	San Adrián de Valdueza	Montes de León (León)	Macizo Galaico-Leonés (Northwest)	42.468/-6.6011	<i>Avd</i>	5
D10	Souto de Moreda	Sierra de O Courel (Lugo)	Macizo Galaico-Leonés (Northwest)	42.5936/-7.1102	<i>Avd</i>	5
D11	Redipuertas	Macizo Asturleonés (León)	Cantabrian Range (Northwest)	43.0118/-5.4679	<i>Avd</i>	5
EU1	Eume I	Fragas do Eume (A Coruña)	Cantabrian Range (Northwest)	43.4051/-8.0376	<i>Avd</i>	4
EU2	Eume II	Fragas do Eume (A Coruña)	Cantabrian Range (Northwest)	43.4137/-8.0546	<i>Avd</i>	5
LHA	Las Huelgas (Arroyo)	Sierra de Segura (Jaén)	Prebetic Range (Southeast)	38.102/-2.7175	ND	5
LHC	Las Huelgas (Covarrón)	Sierra de Segura (Jaén)	Prebetic Range (Southeast)	38.1029/-2.7154	ND	5
PAU	Port de Beceite	Ports de Tortosa-Beceite (Tarragona)	Iberian System (Northeast)	40.8254/0.3675	<i>Avp</i>	5
PC1	Poyos de La Carilarga 1	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8949/-2.8315	ND	5
PC2	Poyos de La Carilarga 2	Sierra del Pozo (Jaén)	Prebetic Range (Southeast)	37.8947/-2.8336	ND	5
PD1	Pico Huevo	Macizo Asturleonés (León)	Cantabrian Range (Northwest)	43.0086/-5.4844	<i>Apd</i>	5
PD2	Cueto de Juan Toribio	Picos de Europa (Cantabria)	Cantabrian Range (Northwest)	43.0096/-5.4831	<i>Apd</i>	5
PXV	Port de Beceite 2	Ports de Tortosa-Beceite (Tarragona)	Iberian System (Northeast)	40.8254/0.3675	<i>Avp*?</i>	2
SIL	Puente de Silvao	Serra da Faladoira (A Coruña)	Cantabrian Range (Northwest)	43.5564/-7.8397	<i>Avv</i>	5
VLB	Las Blancas	Central Pyrenean Range (Huesca)	Pyrenean Range (Northeast)	42.7157/-0.5619	<i>Avi?</i>	1
VLM	La Molina	Eastern Pyrenean Range (Lleida)	Pyrenean Range (Northeast)	42.3366/1.929	<i>Avi</i>	3
VRG	Guara II	Sierra de Guara (Huesca)	Pyrenean Range (Northeast)	42.2993/-0.2223	<i>Avi?</i>	2

Table 2. Parameters of genetic diversity for localities in each genetic group obtained from allelic frequencies (see Methods). PPL: percentage of polymorphic loci at 95% level; H_e : average expected heterozygosity across loci. uH_e : unbiased expected heterozygosity across loci (unbiased for small sample sizes; see Methods). SE: Standard error. NFM: Number of fixed markers. NMI: Average number of markers per individual. NPM: Number of private markers, out of which D were also fixed (i.e. aka Diagnostic markers). N: Number of analysed individuals (with complete genotypic profiles). Localities acronyms follow Table 1. Total averages and by-genetic-group are also shown. It is to be advised that, the table anticipates the genetic groups later defined. ACB and VLB, single-sampled locations were excluded since estimates could not be computed.

Genetic group	Locality	PPL	$H_e \pm SE$	$uH_e \pm SE$	NMI	NFM	NPM (D)	DW	N
Blue	AP1	21.5	0.0623 ± 0.0060	0.0748 ± 0.0072	81.25	79	0	8.86	3
	AP2	23.8	0.0584 ± 0.0055	0.0667 ± 0.0063	91.00	76	1	24.76	4
	AP3	23.4	0.0500 ± 0.0047	0.0571 ± 0.0054	97.00	93	1	10.65	4
	AP4	23.4	0.0506 ± 0.0049	0.0562 ± 0.0054	102.80	92	0	13.40	5
	AP5	22.4	0.0543 ± 0.0051	0.0621 ± 0.0058	95.25	87	0	7.44	4
	PD2	21.3	0.0508 ± 0.0052	0.0581 ± 0.0059	77.20	74	2 (1)	24.61	4
	APG	24.3	0.0638 ± 0.0059	0.0766 ± 0.0071	100.25	93	4 (4)	35.99	3
		22.8	0.0557 ± 0.0053	0.0645 ± 0.0062	92.11	84.57	1.14 (0.71)	17.96	
Purple	PAU	26.2	0.0768 ± 0.0068	0.0878 ± 0.0078	99.00	77	6 (2)	25.41	4
	AC1	25.5	0.0565 ± 0.0052	0.0628 ± 0.0058	112.80	101	1	11.72	5
	AC2	26.4	0.0679 ± 0.0059	0.0776 ± 0.0067	112.00	100	0	5.22	4
	AC3	23.8	0.0507 ± 0.0048	0.0563 ± 0.0053	105.20	96	0	4.76	5
	AC4	31.4	0.0952 ± 0.0074	0.1088 ± 0.0085	128.25	102	3 (1)	25.75	4
	AC5	26.2	0.0680 ± 0.0061	0.0756 ± 0.0068	106.00	87	0	7.19	5
	AC6	30.4	0.0823 ± 0.0066	0.0914 ± 0.0073	116.20	92	0	14.56	5
	AC7	22.8	0.0510 ± 0.0043	0.0612 ± 0.0052	107.33	107	0	3.04	3
	AZ1	24.9	0.0718 ± 0.0063	0.0862 ± 0.0076	109.00	99	0	3.66	3
		26.4	0.0689 ± 0.0059	0.0786 ± 0.0068	110.64	95.66	1.11 (0.33)	11.26	
Green	A00	24.5	0.0639 ± 0.0059	0.0730 ± 0.0067	93.00	74	0	19.48	4
	AV9	27.6	0.0754 ± 0.0065	0.0862 ± 0.0074	99.40	91	1	15.81	4
	CSC	24.5	0.0549 ± 0.0052	0.0610 ± 0.0058	106.20	95	1	6.12	5
	CCB	24.7	0.0564 ± 0.0052	0.0627 ± 0.0058	103.20	91	3	12.81	5
	PC1	24.5	0.0573 ± 0.0053	0.0637 ± 0.0059	100.60	88	3	8.76	5
	PC2	28.3	0.0654 ± 0.0055	0.0727 ± 0.0061	112.20	98	4	13.36	5
	ACB	26.2	0.0597 ± 0.0053	0.0663 ± 0.0059	108.40	96	0	6.54	5
	ACS	27.6	0.0851 ± 0.0072	0.0946 ± 0.0080	99.60	68	2	7.87	5
	AV1	27.2	0.0761 ± 0.0066	0.0870 ± 0.0075	114.00	96	2	11.64	4
	AV2	26.6	0.0640 ± 0.0056	0.0731 ± 0.0064	107.00	96	0	9.03	4
	AV3	27.2	0.0921 ± 0.0076	0.1105 ± 0.0091	95.50	74	0	8.65	3
	AV4	30	0.0853 ± 0.0068	0.0948 ± 0.0076	103.20	78	2	8.00	5
	AV5	29.1	0.0798 ± 0.0066	0.0887 ± 0.0073	112.20	89	0	6.74	5

	AV6	28.7	0.0723 ± 0.0060	0.0803 ± 0.0067	110.20	92	1	6.64	5
	AV7	27.6	0.0680 ± 0.0059	0.0756 ± 0.0066	112.00	97	0	5.44	5
	AV8	26.8	0.0919 ± 0.0079	0.1050 ± 0.0090	97.00	58	5	13.28	4
	LHA	26.8	0.0687 ± 0.0061	0.0763 ± 0.0068	109.00	88	3	8.81	5
	LHC	26.4	0.0697 ± 0.0062	0.0774 ± 0.0069	104.00	82	3	10.41	5
		26.9	0.0714 ± 0.0062	0.0805 ± 0.0070	104.88	86.16	1.57 (0)	9.97	
Orange	AN1	31.4	0.0884 ± 0.0068	0.1010 ± 0.0078	121.50	78	4	8.73	4
	AN3	24.1	0.0577 ± 0.0055	0.0641 ± 0.0061	98.60	81	3	8.68	5
	AN2	27	0.0773 ± 0.0067	0.0859 ± 0.0074	105.00	85	3	11.26	5
	AN4	26.4	0.0962 ± 0.0081	0.1154 ± 0.0097	89.00	59	0	16.60	3
	AN5	23.8	0.0597 ± 0.0057	0.0682 ± 0.0065	95.20	88	0	14.10	4
	AN6	24.3	0.0582 ± 0.0056	0.0647 ± 0.0062	103.80	91	0	8.10	5
		26.1	0.0729 ± 0.0064	0.0832 ± 0.0073	102.18	80.33	1.66 (0)	11.25	
Red	AVA	32.7	0.1115 ± 0.0080	0.1274 ± 0.0091	114.50	83	5	15.29	4
	PXV	21.5	0.0918 ± 0.0084	0.1224 ± 0.0112	86.00	70	1 (1)	5.14	2
	VRG	24.7	0.0957 ± 0.0081	0.1276 ± 0.0108	104.00	91	1	3.94	2
	VLM	23.8	0.0672 ± 0.0061	0.0806 ± 0.0073	105.67	97	0	3.31	3
	AMA	25.3	0.0781 ± 0.0070	0.0893 ± 0.0080	102.00	81	0	4.76	4
	EU1	24.5	0.0660 ± 0.0061	0.0754 ± 0.0070	104.75	91	0	5.59	4
	EU2	24.7	0.0554 ± 0.0051	0.0616 ± 0.0057	103.00	94	1	8.84	5
	SIL	25.3	0.0590 ± 0.0053	0.0656 ± 0.0059	102.60	91	0	7.54	5
	PD1	26.2	0.0710 ± 0.0063	0.0811 ± 0.0072	94.00	74	2	14.02	4
	D01	26.8	0.0737 ± 0.0065	0.0819 ± 0.0072	107.40	86	0	7.19	5
	D02	28.5	0.0735 ± 0.0062	0.0817 ± 0.0069	110.60	91	1	8.35	5
	D03	28.1	0.0728 ± 0.0062	0.0809 ± 0.0069	111.60	93	4	14.19	5
	D04	29.3	0.0889 ± 0.0072	0.0988 ± 0.0080	106.20	80	0	6.54	5
	D05	25.3	0.0750 ± 0.0067	0.0857 ± 0.0077	104.50	86	0	3.52	4
	D06	27	0.0696 ± 0.0060	0.0795 ± 0.0069	100.00	85	1	9.41	4
	D07	27	0.0688 ± 0.0060	0.0764 ± 0.0067	102.80	86	1	7.40	5
	D08	24.7	0.0585 ± 0.0053	0.0669 ± 0.0061	98.40	88	0	13.30	4
	D09	26.2	0.0738 ± 0.0065	0.0820 ± 0.0072	98.40	77	2	5.69	5
	D10	23.6	0.0625 ± 0.0059	0.0714 ± 0.0067	100.75	88	1	5.31	4
	D11	31.6	0.0852 ± 0.0067	0.0947 ± 0.0074	121.40	97	2	14.04	5
		26.3	0.0749 ± 0.0065	0.0865 ± 0.0075	103.79	86.45	1.04 (0.05)	8.17	

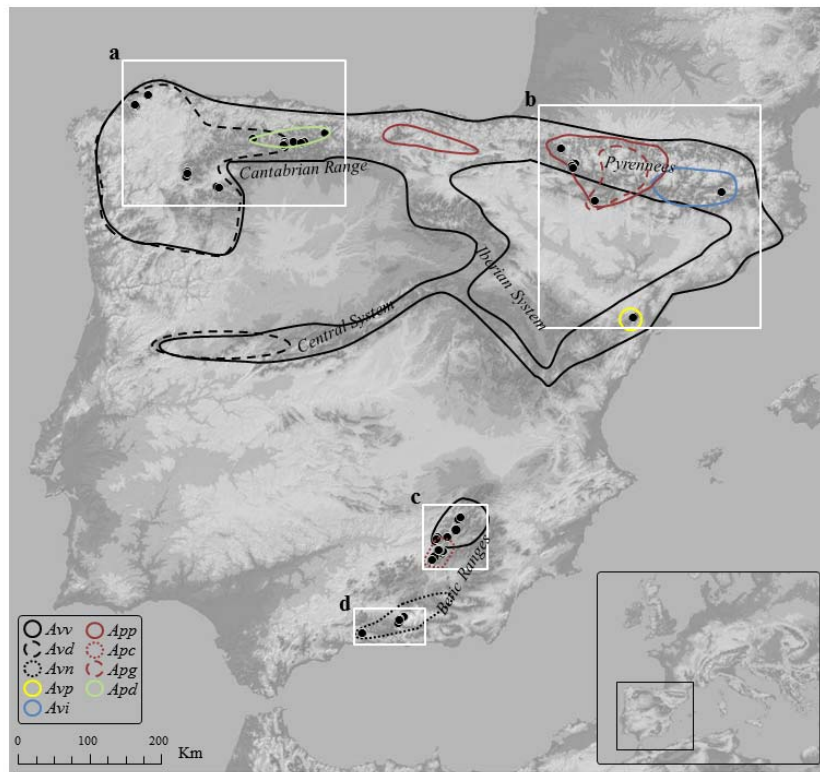


Figure 1

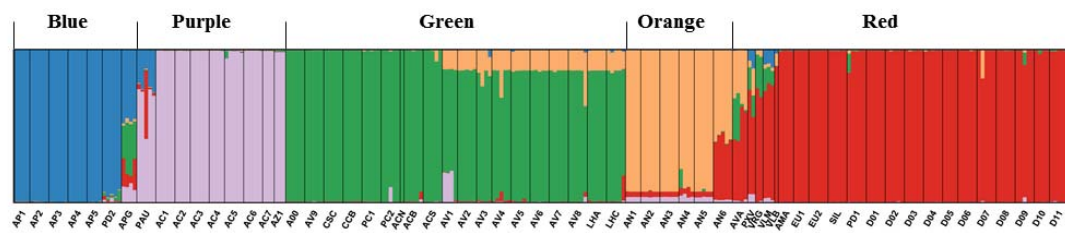


Figure 2

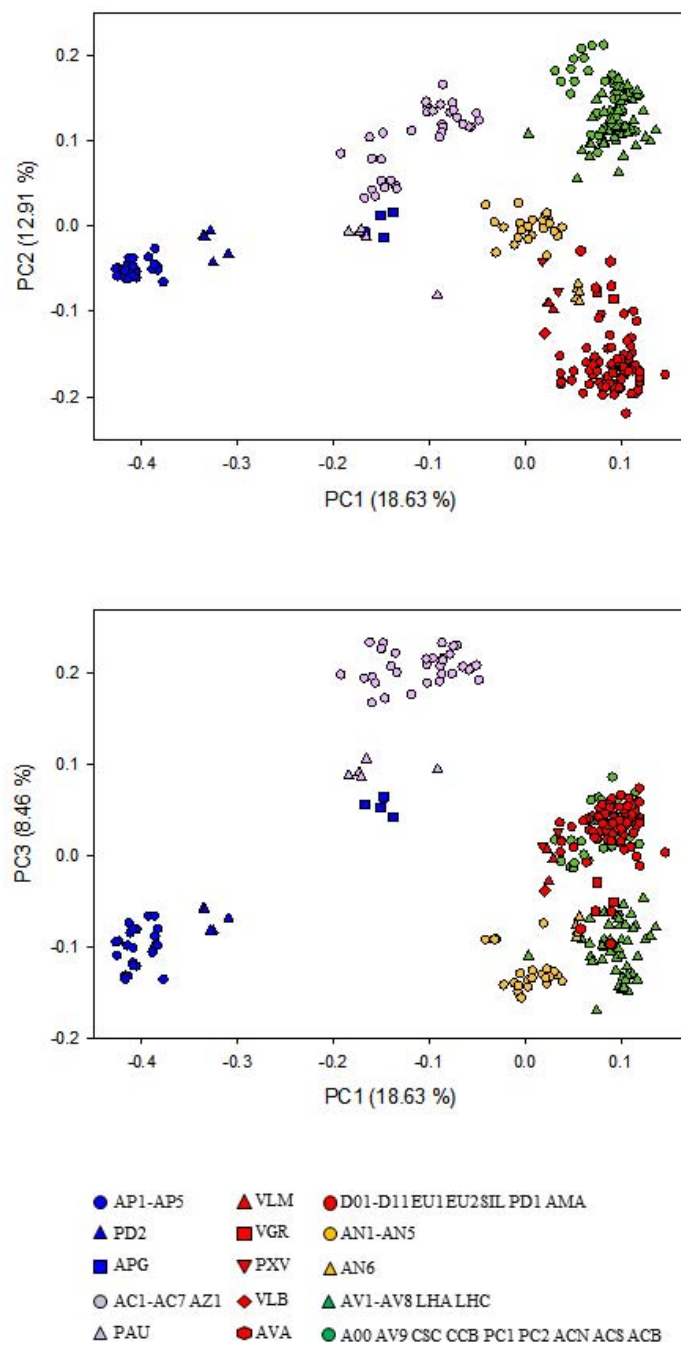


Figure 3

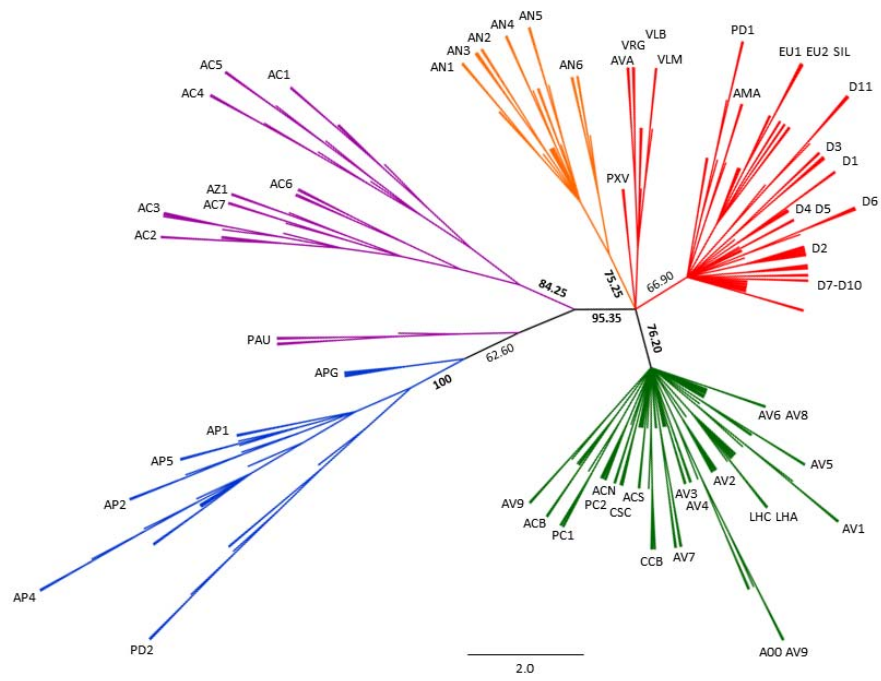


Figure 4

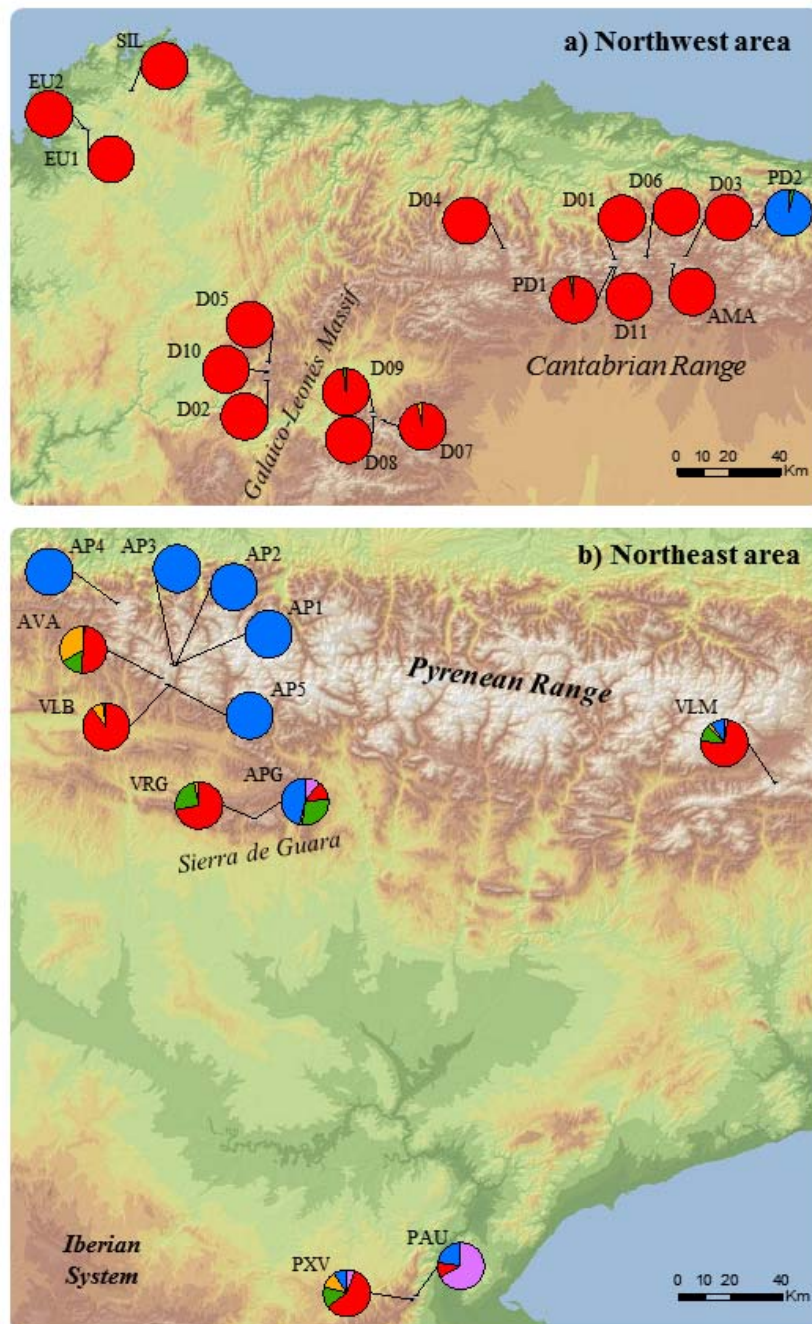


Figure 5

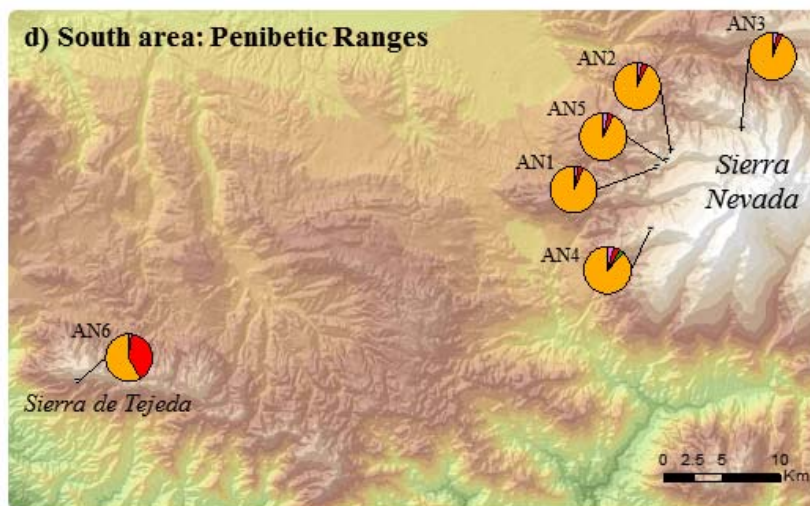
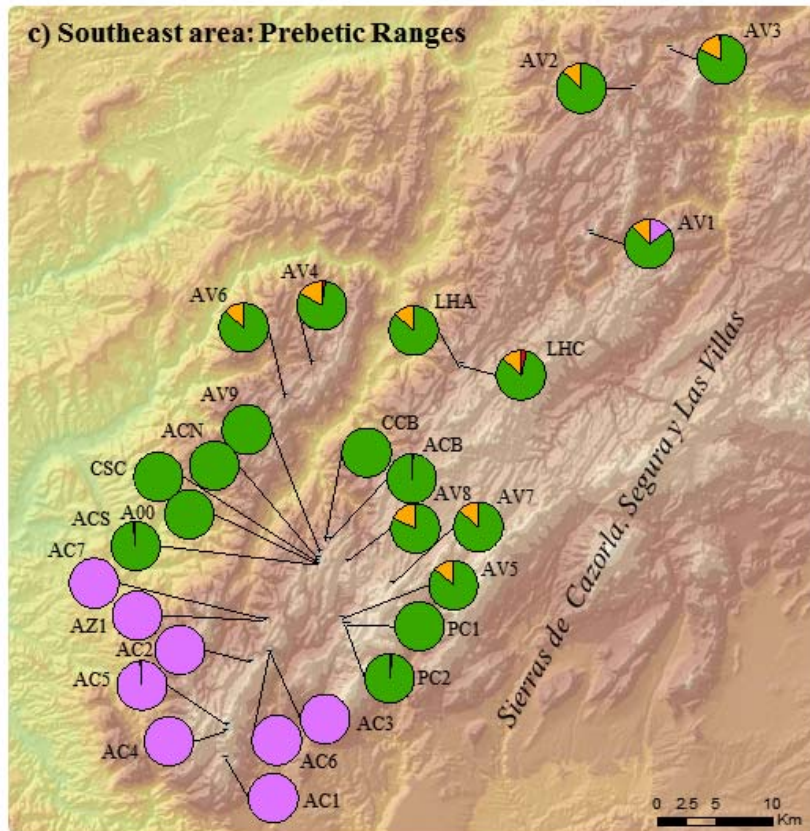


Figure 5 (cont.)

Online Resource 1. Primer combinations used in AFLP analyses of 285 *Aquilegia* individuals sampled across the Iberian Peninsula. NB, Number of Bands; PPB, Percentage of Polymorphic Bands at 99% level; SER, Scoring Error Rate. * A band was considered polymorphic when it showed a total frequency between 0.01 and 0.99. ** Scoring error rate (for 16 individual replicates): (number of misscorings on two completely independent analyses) / (number of markers x number of individuals) x 100.

Primer combination	NB	PPB*	SER (%)**
<i>EcoRI</i> + AGG / <i>Mse</i> + CTT	68	95.77	0.67
<i>EcoRI</i> + AGC / <i>Mse</i> + CTT	85	92.44	0.54
<i>EcoRI</i> + AGG / <i>Mse</i> + CAT	39	96.84	1.27
<i>EcoRI</i> + ACA / <i>Mse</i> + CTT	56	95.58	1.60
<i>EcoRI</i> + AGG / <i>Mse</i> + CAC	43	96.49	1.90
<i>EcoRI</i> + AGC / <i>Mse</i> + CAC	72	94.34	1.34
<i>EcoRI</i> + AGG / <i>Mse</i> + CCC	64	94.89	1.72
<i>EcoRI</i> + ACA / <i>Mse</i> + CAC	47	95.57	1.73
<i>Average</i>	59.37	95.24	1.34

Geographic genetic structure of Iberian Columbines (gen. *Aquilegia*)
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(1) Estación Biológica de Doñana, Agencia Estatal Consejo Superior de Investigaciones Científicas (EBD-AECSIC). Avenida Américo Vespucio, s/n, 41092, Sevilla, Spain.

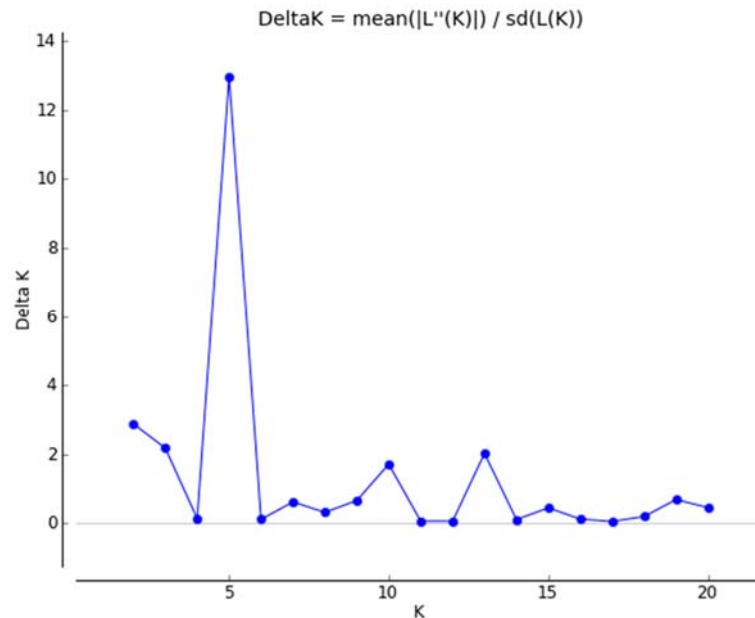
(2) Depto. Biología Animal, Biología Vegetal y Ecología. University of Jaén. 23071, Jaén, Spain.

(3) School of Life Sciences, University of Sussex, Brighton, BN1 9QG, UK.

(4) Depto. Botánica. University of Santiago de Compostela. 15782, Santiago de Compostela, A Coruña, Spain.

(*) Corresponding author: José L. Garrido, e-mail: jlgarrido@ebd.csic.es.

Online Resource 2. Variation in ΔK parameter for the STRUCTURE analysis performed on the whole Iberian *Aquilegia* data set to estimate of the number of genetic clusters (K). The total genetic variation was most likely partitioned into 5 genetic clusters.



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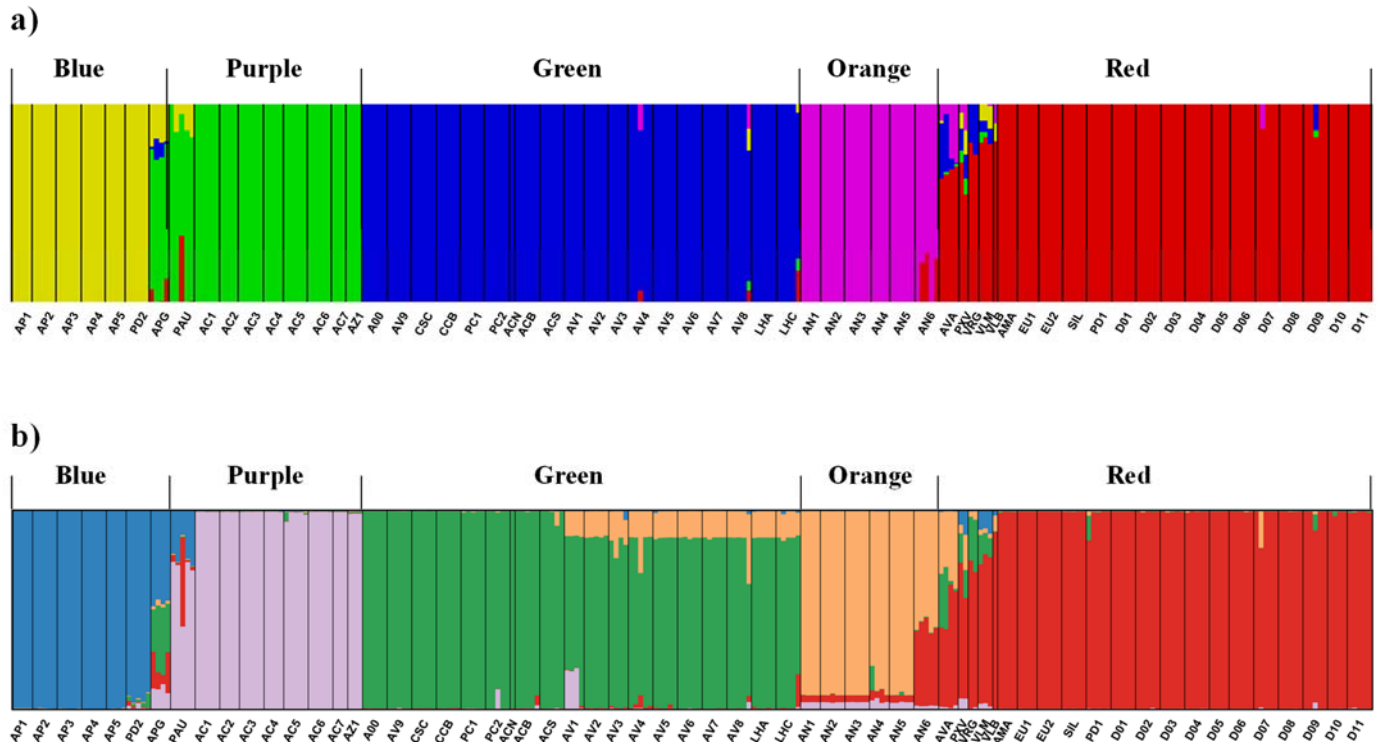
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(4) Depto. Botánica. University of Santiago de Compostela. 15782, Santiago de Compostela, A Coruña, Spain.

(*) Corresponding author: José L. Garrido, e-mail: jlgarrido@ebd.csic.es.

Online resource 3. BAPS analysis results (a) on the genetic structure of the 62 Iberian *Aquilegia* locations based on 474 AFLP multilocus individual profiles. BAPS analyses were performed to confirm and verify the adscription of individuals to the clusters revealed by STRUCTURE. Results show individual memberships to each of the 5 genetic clusters defined by STRUCTURE. Bars with multiple colours indicate admixtures of genotypes from different clusters. Locations are separated by black lines and their acronyms follow Table 1. STRUCTURE results (b) are shown below to allow a direct visualization of the consistence in the individual assignment to clusters among both analyses.



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(2) Depto. Biología Animal, Biología Vegetal y Ecología. University of Jaén. 23071, Jaén, Spain.

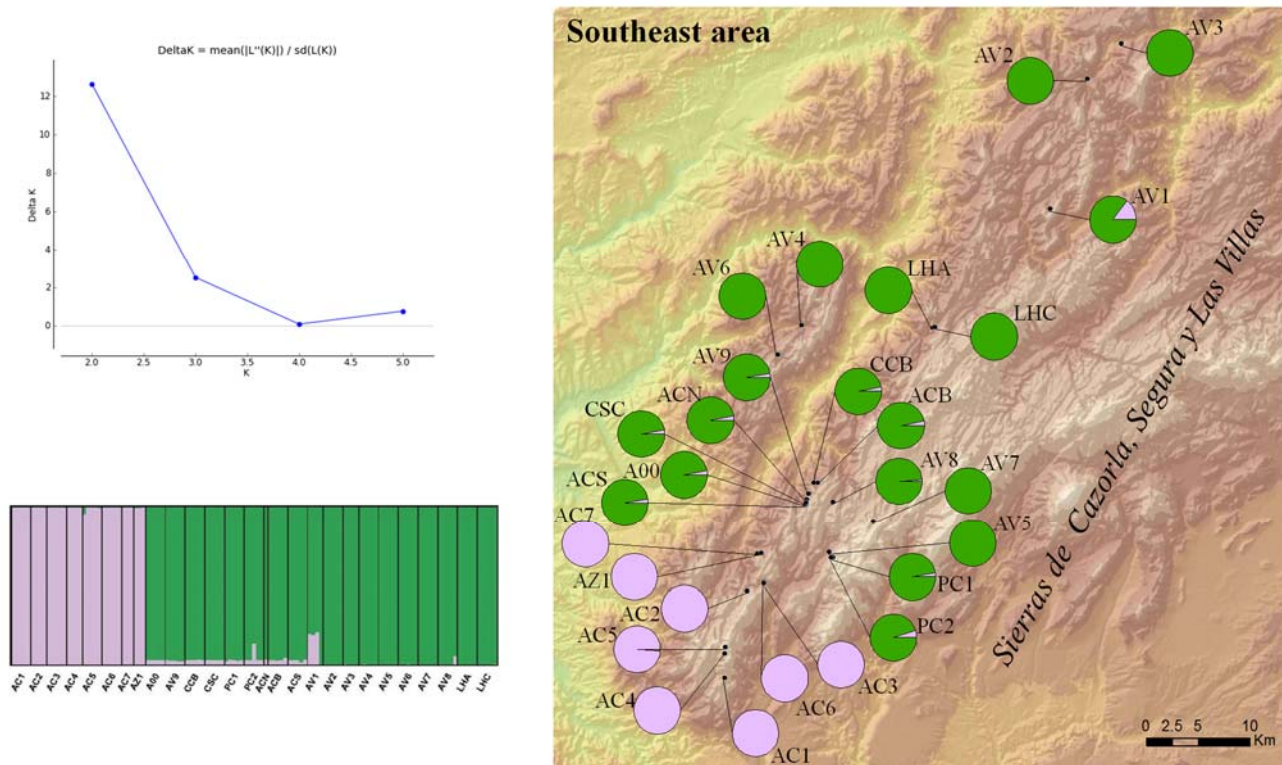
(3) School of Life Sciences, University of Sussex, Brighton, BN1 9QG, UK.

(4) Depto. Botánica. University of Santiago de Compostela. 15782, Santiago de Compostela, A Coruña, Spain.

(*) Corresponding author: José L. Garrido, e-mail: jlgarrido@ebd.csic.es.

Online Resource 4. Separate Structure analysis performed on Prebetic locations

A separated Structure analysis was exclusively performed on Prebetic locations (Southeast area, Figure 1), given the relevance of these mountain systems in terms of Iberian and Mediterranean Biodiversity, and that every known *Aquilegia* location was sampled in this region. We proceed similarly and the number of genetic clusters was estimated as 2 ($\Delta K = 12.61$, $Pr[\ln L = -12910.45]$). Results are shown at Figure ESM_4, specifically, variation in the ΔK parameter, the membership of each sampled individual for the two clusters defined, and the geographic distribution of both clusters across Prebetic Ranges.



Geographic genetic structure of Iberian Columbines (gen. *Aquilegia*) Plant Systematics and Evolution

J. L. Garrido^{1*}, J. M. Alcántara², P. J. Rey², M. Medrano¹, J. Guitián⁴, M. C. Castellanos³, J. M. Bastida², R. Jaime², and C. M. Herrera¹.

(1) Estación Biológica de Doñana, Agencia Estatal Consejo Superior de Investigaciones Científicas (EBD-AECSIC). Avenida Américo Vespucio, s/n, 41092, Sevilla, Spain.

(2) Depto. Biología Animal, Biología Vegetal y Ecología. University of Jaén. 23071, Jaén, Spain.

(3) School of Life Sciences, University of Sussex, Brighton, BN1 9QG, UK.

(4) Depto. Botánica. University of Santiago de Compostela. 15782, Santiago de Compostela, A Coruña, Spain.

(*) Corresponding author: José L. Garrido, e-mail: jlgarrido@ebd.csic.es